AN EXAMINATION OF THE NONINFLAMMATORY ROLE OF ASTROCYTES IN GLUTAMATE EXCITOTOXICITY IN SOD1 G93A ALS MOUSE MODEL

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LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
SOD1 G93A	Superoxide dismutase-1 glycine 93 to alanine
CNS	Central nervous system
GLT-1	Glutamate transporter 1
EAAT2	Excitatory amino-acid transporter 2
CTE-SUMO1	Small ubiquitin modifier 1- conjugated C-terminal fragment
K+-ATP	Potassium-ATP
Ca_{2+}	Calcium
ER	Endoplasmic reticulum
SOCE	Store-operated calcium entry
GluR	Glutamate receptor
WT	Wild-type

SUMMARY

Astrocytes can be found between the vascular and neuronal elements of the central nervous system, where they take up and release molecules in order to maintain a homeostatic microenvironment for optimal neuron growth. Dysregulation of these astrocyte functions can lead to neuronal depolarization, hyperexcitability, excitotoxicity and subsequent neuronal death, characteristic of Amyotrophic Lateral Sclerosis (ALS). We hypothesize that the levels of glutamate and glutamate transporters in astrocytes increase over ALS disease progression and contribute to motor neuron death. Applying inclusion and exclusion criteria to a database comprising findings from over 2,000 papers resulted in data from over 60 papers. Data measuring glutamate concentrations and glutamate transmitter levels from SOD1 G93A (superoxide dismutase-1 glycine 93 to alanine) transgenic ALS mouse models were normalized to wild-type mice and graphed over time. We perform meta-analysis, correlation analysis, and survival analysis to determine the role of astrocytic glutamate regulation in disease progression. We demonstrate that GTL-1, GluR-1, and GluR-2 levels from astrocytes decrease over time. We propose that this trend may be associated with increased extracellular glutamate concentrations and the cellular attempts to maintain homeostasis. The findings of this analysis will give insight into the non-inflammatory role of astrocytes in the pathophysiology of ALS.

CHAPTER 1

INTRODUCTION

Astrocyte dysregulation has long been implicated in many motor neuron degenerative diseases. Astrocytes are the most abundant subtype of glial cells found in the central nervous system (CNS) and make up anywhere from 20-50% of brain volume (Rossi D., 2009). They can be found between the vascular and neuronal elements of the CNS which allows them to take up and release molecules in order to maintain a homeostatic microenvironment for optimal neuron growth (Kawamata et al., 2014). Astrocytes also absorb glucose from the blood and store it as glycogen, turn it into lactate through glycolysis (Rossi D., 2009) or release it if glucose and oxygen supplies are low (Sofroniew & Vinters, 2010). Furthermore, damage to the CNS results in a process known as astrogliosis (Cui et al., 2014). Astrocytes proliferate and migrate to the area of injury. They then surround the injured neurons and undergo cellular hypertrophy to form what is referred to as a glial scar. The glial scar restores blood brain barrier functionality, secludes the injury site, and releases neurotrophic factors to protect against apoptosis (Rossi D., 2009). However, astrocytes can also restrict axon regeneration and secrete neurotoxic and excitotoxic molecules such as pro-inflammatory or cytotoxic cytokines (Fitch & Silver, 2008). Dysregulation of these astrocyte functions can lead to neuronal depolarization, hyperexcitability, excitotoxicity, and subsequent neuronal death, characteristic of Amyotrophic Lateral Sclerosis

(ALS) (Rossi D., 2009).

A majority of ALS research has been focused on the role of astrocytes in the inflammatory response. It is not understood, however, if astrocyte dysregulation proceeds or is a product of the inflammatory response. Little is known about the temporal trends of glutamate excitotoxicity, metabolic homeostasis, and intracellular calcium signaling in disease progression (Li, 2015, Ngo, 2015, Kawamata, 2014). A study found there were no significant physiological benefits from increasing glutamate transporter 1 (GLT-1) levels in SOD1 mice, and it is believed that this is due to the occurrence of other critical pathophysiological events at the time of treatment (Li, 2015). An understanding of the changes in glutamate levels over disease progression is needed to support this hypothesis. Such research could help identify the optimal time for different types of treatment interventions.

Using a database of over 2,000 papers, we propose to run meta-analysis and correlation analysis on data from papers measuring the concentrations of molecules involved in glutamate excitotoxicity, metabolic homeostasis, and intracellular calcium signaling in astrocytes from G93A SOD1 mice. Increased levels of glutamate and reduced levels of excitatory amino-acid transporter 2 (EAAT2) have been found in the CNS of ALS patients, suggesting EAAT2 dysfunction is involved in the disease progression (Li, 2015). The concentrations of glutamate, glutamate regulators, glutamate transporters, and glutamate receptors will be compared to determine the presence of significant temporal trends in disease progression. The findings of these analyses will give

insight into the non-inflammatory role of astrocytes in the pathophysiology of ALS.

CHAPTER 2

LITERATURE REVIEW

Glutamate Excitotoxicity

Astrocytes express glutamate transporters, GLT-1 in mice and EAAT2 in humans, which are responsible for most of the glutamate uptake in the CNS. Increased levels of glutamate and reduced levels of EAAT2 have been found in the CNS of ALS patients, suggesting EAAT2 dysfunction is involved in the disease progression (Li et al., 2015). The only FDA-approved treatment for ALS is an anti-glutamatergic compound called Riluzole but its benefits are mild and little is known about its mechanisms. Because of this, many studies have focused on finding ways to increase GLT-1 levels in astrocyte to increase glutamate reuptake. However, few have been successful. One such study (Li et al., 2015) used intraspinal injections of a GLT-1 vector in SOD1 G93A mice to study the physiological benefits of a potential gene delivery treatment. The reuptake of glutamate by astrocytes was measured, as well as motor neuron survival and *in vivo* information, such as hind limb strength and percent survival. The study found no physiological benefits from increasing intraspinal GLT-1 levels in SOD1 mice. It is believed that the main cause of the treatment failure was that the injections only began at 110 days of age, the age of

ALS onset in SOD1 mice. If GLT-1 levels are becoming low at any point before onset, inducing GLT-1 treatment at onset may be too late effect a change in the

physiology. Additionally, GLT-1 is not the only component in the glutamate reuptake process. The benefits of a GLT-1 treatment could be optimized by an understanding of the temporal changes of glutamate and GLT-1 levels over the disease progression, as well as the interaction between other glutamate regulating molecules.

One such molecule is small ubiquitin modifier 1- conjugated C-terminal fragment (CTE-SUMO1) which accumulates in spinal cord during disease progression (Foran, 2014). It was suspected that CTE-SUMO1 was affecting the localization of function of EAAT2 in astrocytes. Using primary astrocyte cultures of G93A mice, Foran et al. (2014) forced EAAT2 sumoylation *in vitro*. Increased intracellular clusters of EAAT2 and almost complete removal of EAAT2 from the plasma membrane were observed. This lead to reduced reuptake of extracellular glutamate. This study showed that, due to protein regulation within the cell, increasing EAAT2 levels doesn't necessarily lead to the desired increases in glutamate reuptake, as observed in Li et al. (2015). Since the study was done *in vitro,* however, there is still a question of its efficacy. Localization *in vitro* can be very different than localization *in vivo* (Foran et al., 2014) and the results may not translate to *in vivo* disease progression. A broader, more comprehensive study of the interactions of all the components of glutamate regulation needs to be studied *in vivo*.

Metabolic Homeostasis

ALS-causing genes are also linked to lipid homeostasis, glucose homeostasis, mitochondrial formation, ATP production, and other metabolic functions (Ngo and Steyn, 2015).

Extracellular concentrations of ATP in the CNS regulate activation and migration of immune and glial cells and increase in response to trauma and inflammation. These concentrations effect metabotropic (P2Y) and ionotropic (P2X) receptors in the nervous system. Activation of the P2X7 receptor, in particular, causes an increase in intracellular calcium (Gandelman et al., 2010). This activation, however, requires a 100 micromolar concentration of ATP and is believed to only arise under pathological conditions. In comparison, the P2X3 and P2X2/3 receptors require only 1 micromolar of ATP for activation (Lewis et al., 1995). In astrocytes it can cause pro-inflammatory signaling which causes an increased production of nitric oxide and various chemokines (Gandelman et al., 2010). It has been found that G93A astrocytes degrade ATP at a faster rate than non-transgenic astrocytes and display ATP-dependent proliferation. It has also been shown that high extracellular concentrations of ATP can cause non-transgenic astrocytes to induce motor neuron death (Gandelman et al., 2010). Mice with the SOD1 G93A mutation experience hypermetabolism and defects in glucose metabolism leading to significant weight loss. ALS metabolic dysfunction can have affects throughout the body and can be observed in the adipose, liver, and muscle tissues. Evidence shows that a complicated cycle of bioenergetic deficits may worsen disease progression over time (Ngo and Steyn, 2015). Ngo and Steyn (2015) studied the potential of potassium-ATP (K+-ATP) ion channels as a potential target for treatment of ALS. They specifically note the use of diazoxide, a small molecule that activates K+-ATP channels, in the treatment of other neurodegenerative diseases, such as Parkinson’s and Alzheimer’s. Similar to

glutamate, the temporal trends of metabolic homeostasis need to be determined in order to optimize treatments.

Calcium Homeostasis

A third non-inflammatory function of astrocytes is maintaining intracellular calcium (Ca_{2+}) homeostasis. Intracellular Ca_{2+} plays a critical role in cell signaling and is tightly regulated in order to maintain homeostasis. Astrocytes are able to signal neurons by Ca_{2+} dependent release of glutamate (Rossi and Volterra, 2009). Intracellular concentrations of Ca_{2+} are characteristically elevated in response to pathological signaling. When the IP3 receptor is activated, Ca_{2+} is released from the endoplasmic reticulum (ER). The depletion of Ca_{2+} for the ER activates the store-operated calcium entry (SOCE) in order to maintain homeostatic conditions. However, oxidative stress can cause enhanced SOCE which leads to an overload of Ca_{2+} in the ER. This overload is closely associated to cell death pathways found in ALS progression (Kawamata and Manfredi, 2010). The excitotoxic glutamate response of astrocytes relies on intracellular concentrations of Ca_{2+} but has also been found to be accompanied by the cytokine TNF-alpha. The blockage of the formation of TNF-alpha and ER Ca_{2+} overload have a significant negative effect on astrocyte glutamate release (Kawamata and Manfredi, 2010, Rossi and Volterra, 2009).

Kawamata et al. (2014) found significantly increased levels of a modified protein involved in ER calcium release in ALS motor neuron cultures. This lead to significantly higher levels of intracellular calcium in the cultures. When they inhibited basal exocytosis in wildtype mice, however, there was no effect on

motor neuron survival. This suggests there are factors at play other than just Ca_{2+} homeostasis dysregulation. They did not, however, look at end-stage neurons. Since the temporal trends of Ca_{2+} and glutamate regulation are not well understood there is a chance of delayed effects that can't be seen until the end-stage of the disease. Additionally, because glutamate, ATP, and Ca_{2+} homeostasis physiologically overlap, the changes in each should not be studied in isolation. All three categories should be studied over time *in vivo* to understand how the whole system interacts and changes throughout disease progression. Due to the large number of variables and the increased complications of *in vivo* studies, however, a single, all-encompassing experiment would not be feasible.

Meta-Analysis

The extent of research needed in this area naturally lends itself to a meta-analysis. Meta-analysis uses a collection of data from a large number of published papers to study trends between studies rather than within a single study. In Irvin et al. (2015) they used meta-analysis to determine temporal trends in cellular respiration, oxidation, and calcium homeostasis in SOD1 G93A mouse models. They aggregated data from over 45 papers that met a specified set of inclusion/exclusion criteria and ran statistical analysis to find overarching trends spanning the whole life of a G93A mouse. The data included 15 different *in vivo* measures from four major categories; calcium regulation, mitochondrial mechanisms, cellular respiration, and oxidative regulation. They found that cellular respiration and corresponding ATP production is depressed starting from birth. Due to the nature of a meta-analysis study, however, there is a lot of

information and data excluded from the analysis. All the data must be aggregated in order to run statistical analysis. This requires combining data points from multiple different experiments. All the data points must fit a certain set of exclusion and inclusion principles in order to be included to ensure the quality of the statistical analysis. Because of this, many papers with useful data are not included. Much of ALS research is done *in vitro* using cell cultures as this is the faster and easier first step. However, this does not accurately reflect the disease onset and progression found in patients. Using meta-analysis allowed Irvin et al. (2015) to analyze data from the more accurate *in vivo* models in a shorter time frame.

The current study will address the lack of understanding of the temporal changes in astrocyte regulation of glutamate homeostasis. It will also consider the *in vivo* relationships and interactions of regulating molecules in the categories. This will be done using meta-analysis of published papers from a database of over 2,000 papers on ALS G93A mouse model research. Statistical analysis will be run to determine significant changes in the intracellular levels of glutamate and other molecules in astrocytes over disease progression. This research will help expand the body of knowledge on the mechanisms of ALS disease progression, specifically the non-inflammatory role of the astrocytes and will potentially contribute to finding optimal times for the different types of treatment intervention.

CHAPTER 3

METHODS

Keyword Searches

Keyword searches included a broad list of terms for each ALS subtopic explored. A list of these terms and which area they belonged to is found in Table 1. These terms were used to identify articles with potential data points.

Inclusion Exclusion Criteria

Inclusion and exclusion criteria were created with the goal of limiting the number of papers to those with only useable data for analysis. Only data that met all the inclusion requirements and met none of the exclusion requirements was used. Studies using the transgenic SOD1 G93A mouse model to measure glutamate, glutamate transporters (GLT-1), glutamate receptors (GluR1 and GluR2), and intracellular calcium (Ca_{2+}) in astrocytes were included in this study. Only data that was normalized to an age-matched wildtype (WT) mouse values or data that had corresponding age-matched WT mouse values that could be used for normalization were included. Any studies using low copy G93A mouse models were excluded. Low copy mice have longer lifespans and show symptom onset later than high copy mice. To accurately study changes over disease progression, only one copy number could be used. Low copy mice were excluded because they are the less common of the two models. A study was considered to use low copy mice if it was explicitly stated in the paper. Any data without identifiable

time points or cell extraction times, data without corresponding age-matched WT values, data from mice given a treatment, or data not from astrocytes was excluded in this study.

Analysis of Data

Quantified data was used to make ratios of SOD1 concentrations normalized to WT that were graphed over time. Measures of intracellular GTL-1, GluR1, GluR2, glutamate, and calcium were analyzed. The data points were aggregated into three disease stages; pre-onset (0-96 days), post-onset (97-116 days), and end stage (117+ days). These brackets were determined by finding the average age of onset and age of death of the mice used in the studies (97 and 117 days, respectively). Power analyses were performed on the data groups to determine minimum sample sizes needed to draw valid statistical conclusions. To reach an appropriate sample size, six data points were extrapolated from the post-onset glutamate data. To determine statistical significance of protein levels between disease stages an ANOVA was run using the Bonferroni correction. To compare protein levels in G93A from WT a Student's t-test was run at each disease stage.

CHAPTER 4

RESULTS

A database of over 2,000 articles on ALS in the G93A SOD1 mouse model was narrowed down to 65 papers on glutamate regulation in astrocytes using keyword searches (Fig. 1). Applying the inclusion and exclusion criteria

resulted in 219 useable data points. Keyword searches were also used to identify 77 and 75 papers for ATP and calcium data, respectively.

There was no significant difference in glutamate or GluR2 concentrations in astrocytes over disease progression (Fig. 2 and 3). Glutamate and GluR2 concentrations were also not significantly increased from WT concentrations at any disease stage. At end-stage, GLT-1 levels in astrocytes were significantly lower than end-stage WT values ($p=0.036$) and pre-onset G93A values ($p=0.0061$) (Fig.4). GluR1 concentrations in astrocytes were significantly decreased from post-onset levels ($p=0.013$) (Fig. 3). Intracellular calcium concentrations in G93A were significantly increased from WT at pre-onset ($p= 0.0425$) (Fig. 5).

CHAPTER 5

DISCUSSION

Astrocytes do not Compensate for Increased Glutamate Levels

It has been proposed that the hyperexcitability of motor neurons is caused by overstimulation by the main excitatory neurotransmitter, glutamate, which leads to a large influx of Ca_{2+} into the cell (Do-Ha, 2017). Motor neurons possess little ability to counteract the effects Ca_{2+} influx so overstimulation easily leads to excitotoxicity and cell death. Astrocytes play a critical role in reabsorbing excess extracellular glutamate and maintaining homeostasis for the motor neurons. This study found, however, that the concentrations of glutamate do not significantly increase in astrocytes over disease progression (Fig. 2). This suggests that the increased levels of glutamate found in the CNS of ALS patients are not being

reabsorbed by the astrocytes and are being left in the extracellular fluid to over stimulate the postsynaptic neurons.

It is still unknown as to what causes the glutamate to collect within the CNS to begin with. One possible explanation is that, prior to onset of the disease, the astrocytes fail to reuptake the extracellular glutamate at the proper homeostatic rate. Another explanation is that the motor neurons, or some other cell, release glutamate at a significantly higher rate than normal. The astrocytes, however, continue to reabsorb at a normal rate which causes the glutamate to accumulate over time. Since the glutamate concentrations in the astrocytes are not significantly different from the levels found in WT (Fig. 2) at any time bin, it is likely that the astrocytes are not causing the initial problem. Why the astrocytes are not responding to the accumulation of glutamate is a point for further review.

Astrocyte GLT-1 a Potential Target of ALS Treatment at End-stage

Due to the moderate success of Riluzole, a compound that is thought to increase glutamate reuptake, there has been significant focus on finding ways to increase the reuptake of glutamate by astrocytes (Do-Ha, 2017). Additionally, post-mortem studies of ALS tissue and mice have found reduced levels of EAAT2 and GLT-1, respectively (Rothstein, 1995, Pardo, 2006). It is believed that decreased GLT-1 levels are contributing to the accumulation of extracellular glutamate and increasing the levels could help prevent the hyperexcitability of the motor neurons. Few experiments have had any notable success, however, due to the complex interaction and timing of the pathophysiology (Li et al. 2015). This study found that GLT-1 levels in astrocytes only decrease significantly from post-

onset to end-stage (Fig. 4). This suggests that increasing astrocytic GLT-1 levels before end-stage may increase glutamate reuptake and prolong survival.

Astrocyte GluR1 is a Potential Target for ALS Treatment at Pre-onset

The influx of Ca_{2+} caused by the overstimulation of motor neurons leads to cell death as Ca_{2+} is involved in many apoptotic pathways. The permeability of glutamate receptors is determined by GluR subunits. Glutamate receptors with GluR1 subunits are highly permeable to Ca_{2+} and causes the mobilization of intracellular Ca_{2+} when activated (D'Antoni et al., 2011). GluR2 lacking glutamate receptors are highly permeable to Ca_{2+} (Grosskreutz, 2010). Studies have shown that a lack of GluR2 increased motor neuron degeneration (Van Damme et al., 2005) in mice and increasing GluR2 levels in motor neurons prolonged survival of mice (Tateno et al, 2004). While decreased GluR2 levels were not observed in ALS astrocytes in this study, there was a decrease in GluR1 levels at end-stage when compared to pre-onset (Fig. 3). Intracellular Ca_{2+} levels also decreased from elevated levels at pre-onset to WT levels at end-stage. This suggests that reducing GluR1 levels in astrocytes at pre-onset could reduce intracellular Ca_{2+} levels and Ca_{2+} dysregulation effects. Additionally, GluR1 is elevated from WT at post-onset, though this difference wasn't found to be significant. This lack of significance may be attributed to the small sample size of this time bin. Due to a limited number of data points quantified for intracellular Ca_{2+} that fell within the post-onset time bin this study was unable to examine the relationships of Ca_{2+} and the GluR subunits between 97 and 116 days.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The purpose of this research was to help further understand the role of astrocytes and glutamate reuptake in the pathophysiology of ALS. Through meta-analysis it was found that intracellular glutamate does not change over time suggesting that the astrocytes are not responding to the increase in extracellular glutamate. GLT-1 levels were found to be decreased only at end-stage meaning GLT-1 treatments should be administered before 117 days of age in mice. Additionally, normal intracellular calcium levels correspond with decreased GluR1 levels. This indicates that lowering GluR1 levels in astrocytes could help improve calcium homeostasis and prolong cell survival. Altogether, this study finds that astrocyte function is failing to protect the motor neuron microenvironment in unexpected ways. Understanding these failings will help move ALS research closer to uncovering more effective treatments for the disease.

Moving forward, the role of astrocytes in calcium homeostasis will be examined with the same methods. The levels of various cytokines involved in the functions and uses of intracellular calcium will be compared across time bins to obtain a better understanding of why intracellular calcium levels are significantly increased at pre-onset.

The hope for this research is to guide future studies towards more effective forms of ALS treatment. The lack of promising results from GLT-1 treatments in mice demonstrates this need. Timing of treatments is critical in clinical settings and these results suggest the GLT-1 treatments should be ministered prior to end-stage, whereas GluR treatments should begin as early as possible during pre-onset. The cross-talk between the glutamate excitotoxicity and calcium homeostasis pathways should also be examined more in depth.

APPENDIX A

FIGURES AND TABLES

Table 1. **Keywords for "*Astrocytes*" and terms associated with each subtopic.**

Subtopic	Terms
Glutamate Excitotoxicity	nitric oxide, NO* conc*, synt*, glutamate, glutamate* conc*, extracellular NO synt* conc, extracellular NO synt* level, GLT1 density in hippocampus, GLT1 density in striatum, GLT1 density in M1Cx, GLT1 density in spinal cord, GLT1 density in S1Cx, GLT1* transporter*, GluRA, GluR1, GluR2, GluR3, GluR4

The subtopics were broad topics that were used to categorize articles discussing ALS studies. The keywords were used in searches in the database to determine article numbers and potential data points for each subtopic.

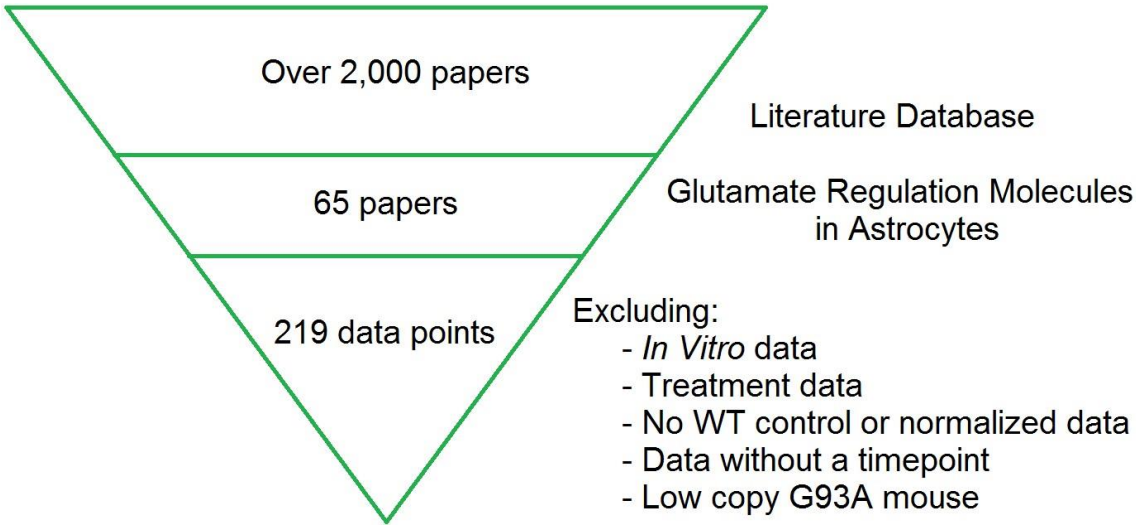

Figure 1. **Inclusion and exclusion criteria.** Inclusion/exclusion criteria were created with the goal of limiting the number of papers to those with only useable data for analysis.

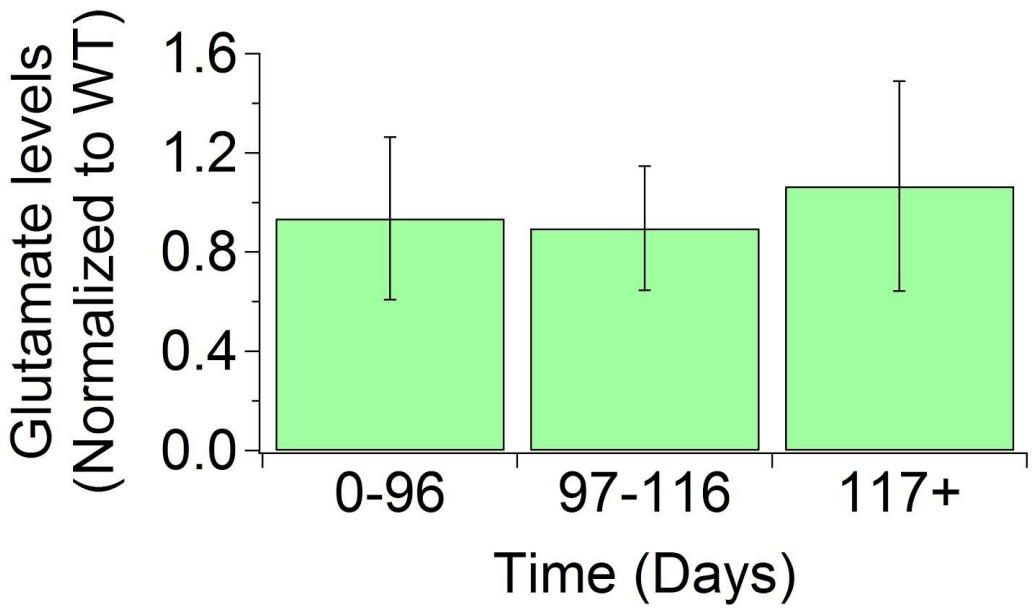

Figure 2. **Glutamate levels in astrocytes over ALS disease progression.**

Glutamate average ratios normalized to wild-type in three stages: 0-96 days, 97-116 days, and 117+ days. There was no significant change in glutamate levels between any of the time bins.

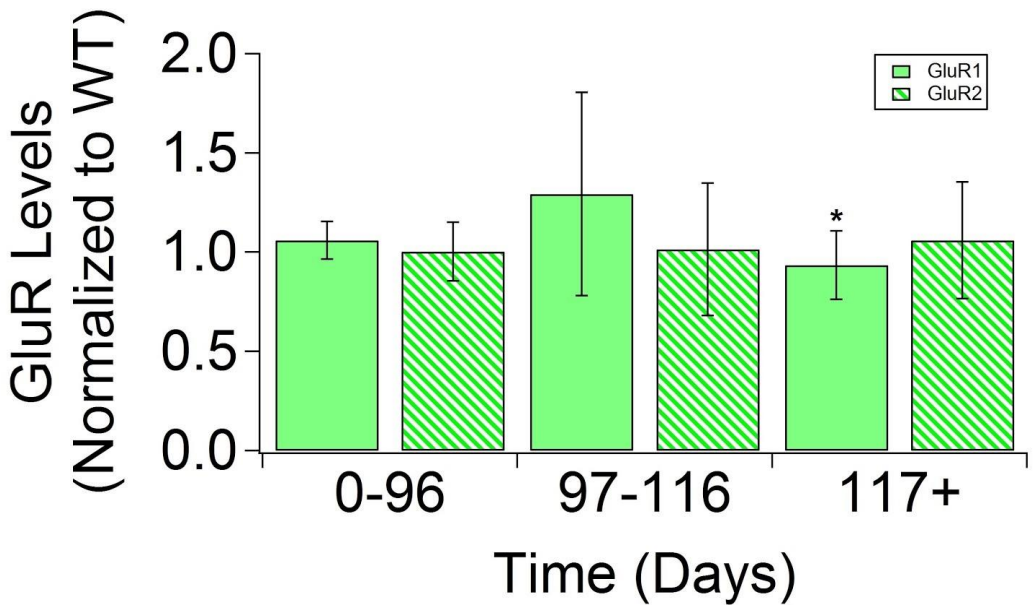

Figure 3. **GluR Complex levels in astrocytes over ALS disease progression.**

GluR1 and GluR2 average ratios normalized to wild-type in three stages: 0-96 days, 97-116 days, and 117+ days. There was no significant change in GluR2 levels between time bins. GluR1 at 117+ days was significantly decreased from 97-116 (*p=0.013).

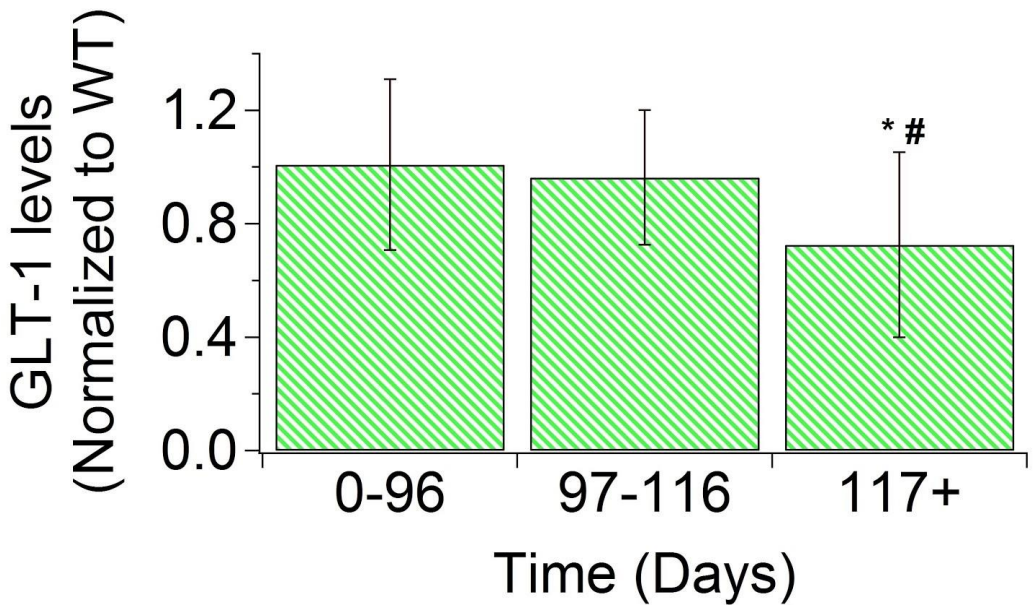

Figure 4. **GLT-1 levels in astrocytes over ALS disease progression.** GLT-1 average ratios normalized to wild-type in three stages: 0-96 days, 97-116 days, and 117+ days. GLT-1 is significantly decreased from 0-96 days (*p=0.036) and from WT (#p=0.0061) at 117+ days.

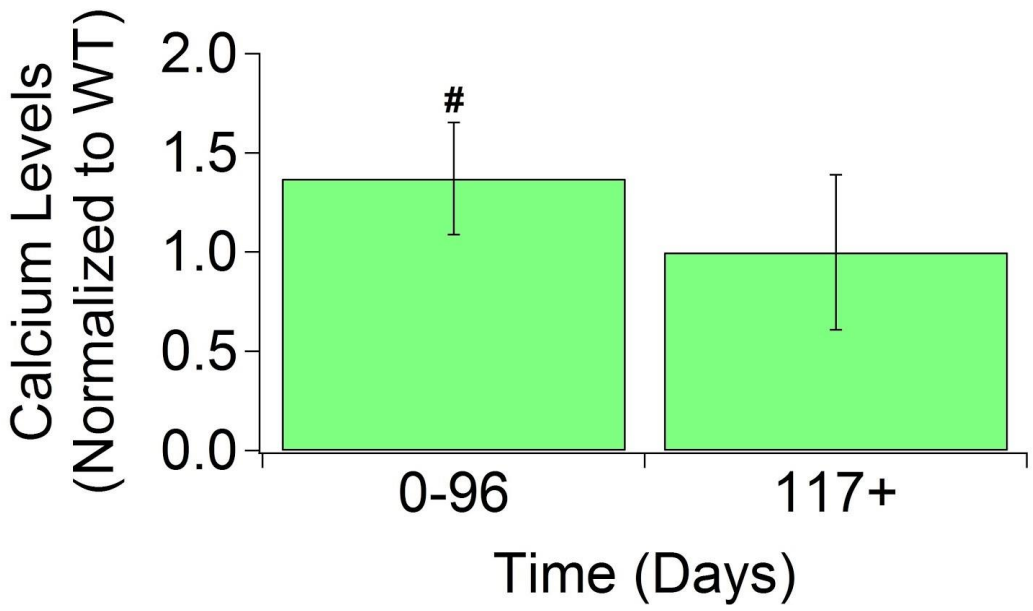

Figure 5. **Calcium levels in astrocytes over ALS disease progression.**

Intracellular Ca_{2+} average ratios normalized to wild-type in two stages: 0-96 days and 117+ days. Ca_{2+} at 0-96 days was significantly increased from WT (#p=0.0425) but not at 117+ days.

APPENDIX B

PAPERS USED IN STUDY

Glutamate

Alexander, G.M., Deitch, J.S., Seeburger, J.L., Valle, L.D. & Heiman-Patterson, T.D. (2000) Elevated cortical extracellular fluid glutamate in transgenic mice expressing human mutant (G93A) Cu/Zn superoxide dismutase. Journal of neurochemistry, 74, 1666-1673.

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GLT-1

Alexander, G.M., Deitch, J.S., Seeburger, J.L., Valle, L.D. & Heiman-Patterson, T.D. (2000) Elevated cortical extracellular fluid glutamate in transgenic mice expressing human mutant (G93A) Cu/Zn superoxide dismutase. Journal of neurochemistry, 74, 1666-1673.

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